National Ophthalmic Disease Genotyping Network
eyeGENE®

PROTOCOL FOR DNA EXTRACTION [POST-CELL LYSIS] AND STORAGE FROM WHOLE BLOOD, INCLUDING LOW-NUCLEATED OR COMPROMISED WHOLE BLOOD

Approved by: _____

Date effective: _____March 29, 2012_____

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Version 2

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Purpose:
This protocol continues the process of purification of genomic DNA from fresh whole blood post-cell lysis using the Gentra Puregene Blood Kit. If blood lysate is compromised in any way (i.e. contains blood clots) or has an initial volume of 3 mL or less, use glycogen (as detailed below) to maximize DNA yield.

Materials:
Falcon 50 mL Tubes (Biosciences INC Cat #: 352070)
1.5 mL Screw Cap Micro Tubes with O-ring (Sarstedt Cat #: 72692005)

Equipment:
Centrifuge (Thermo Electric Corporation INC, Cat #: 75004377)
180 Series Waterbath (Precision INC, Cat #: 51221073)
Sprout® Mini-Centrifuge (Heathrow Scientific LLC, Cat #: HD1000AU)
NanoDrop Spectrophotometer (NanoDrop INC, Serial #: C764)
Vortex Genie (Daigger INC, Cat #: 22220A & 3030A)

Reagents:
RNase A (Qiagen INC Cat #: 158389)
Protein Precipitation Solution (Qiagen INC Cat #: 158389)
100% Isopropanol (2-propanol) (VWR International, Inc. Cat #: JT9084-1)
Ethyl Alcohol Denatured (Fisher Scientific Cat # A407500)
DNA Hydration Solution (Qiagen INC Cat #: 158389)
Glycogen Solution (Qiagen INC Cat #: 158930)

Procedure:
1. Preheat the drybath to 37ºC

2. Print the Manual DNA Extraction Worksheet. If there are samples that need to be extracted using glycogen in the batch, check the box that glycogen will be used during extraction and be sure to fill in the appropriate blanks and checkpoints related to glycogen in the following procedure.

3. Login to the eyeGENE® database, enter the six-digit blood ID from the shipping form and click PROCESS on the Specimen Processing page. DNA quantity is usually split between two tubes (d1 and d2). Enter the volume of DNA Hydration Solution the DNA sample will be re-suspended (see Part IV, Table 1 of DNA Extraction Worksheet). Assign a storage position to each DNA aliquot and enter the location on the eyeGENE® database and the DNA Extraction worksheet. Make a note in the patient's adjunct box in the database that sample was extracted using glycogen, if applicable.

4. Print DNA labels (3 copies each of the d1 and d2 labels) from the eyeGENE® database. Place one copy of each d1 and d2 labels on the DNA Extraction

Worksheet. If applicable, note which samples will be extracted with glycogen on the worksheet.

5. Repeat steps 1-3 for all of the eyeGENE® samples that will undergo DNA extraction in that batch. Remember to leave one space on your worksheet for a blank tube that will act as a negative control.

6. Bring the DNA extraction worksheet and the 2nd print out of d1 and d2 labels to the lab bench. Label the corresponding empty 50 mL tubes with d1 and d2 eyeGENE® IDs. If applicable, write "Glycogen" clearly on the sides and tops of all tubes of the samples that will be extracted using glycogen to distinguish them from the samples that will be extracted normally. In addition, collect two empty, 50 mL tubes and label them as negative controls.

7. Using color labels, color code the samples using a separate color for each patient sample. Put one color label on the top of the tube and one on the side of the tube. Confirm that the color code on the sample tubes matches the color code on the DNA Extraction worksheet (Checkpoint A of DNA Extraction Worksheet). It is not necessary to color code the negative control.

8. Confirm that the order on of eyeGENE® samples on the DNA Extraction Worksheet matches the order of samples on the rack (Checkpoint B of DNA Extraction Worksheet).

9. Fill one of the empty tubes used for the negative control with deionized water. Use the same volume of deionized water as the smallest sample in the extraction batch as the starting volume for the negative control. For example, if the smallest cell lysate volume in the batch is 4 mL, use 4 mL of deionized water as the negative control and extract as if the blank was a 4 mL sample. Using the smallest volume possible for the negative control will help to conserve reagents. The negative control should be processed normally.

   ***Note: If 50% or more of the tubes in the extraction batch require glycogen to be extracted, include glycogen in the negative control. If less than half of the samples in the extraction batch require glycogen, do not include glycogen in the negative control.*

** If possible try to group samples by initial blood volumes. For example, group the samples with 4 mL of blood together, the samples with 6 mL of blood together, etc.

CAUTION: Make sure the pipette tip to be used in reagents does not touch any side of the 50 ml tube during the process to avoid cross contamination of reagents and whole blood.

RNAse Treatment

10. Add the appropriate amount of RNase A Solution to the 50 mL sample tube containing the cell lysate (see Part IV, Table 1 of Extraction Worksheet).

11. Mix the sample by inverting the tube 25 times followed by incubation at 37°C for 15 minutes.

Protein Precipitation

12. Allow the samples to cool to room temperature (10-15 min.). In the meantime, turn the drybath up to 68°C.

13. Add Protein Precipitation Solution to the cooled samples (see Part IV, Table 1 of DNA Extraction Worksheet).

14. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate.

15. Centrifuge at 2,000 x g for 5 minutes (Program 1). The precipitated proteins will form a tight dark brown pellet. If the protein pellet is not tight, repeat Step 11 followed by incubation on ice for 5 minutes and then repeat Step 12.

DNA Precipitation

16. Add the appropriate amount of 100% Isopropanol (2-propanol) (see Part IV, Table 1 of DNA Extraction Worksheet) to the empty, pre-labeled, sterile 50mL tubes. For those samples that need to be extracted using glycogen, add the appropriate amount of Glycogen Solution (see Part IV, Table 1 of DNA Extraction Worksheet) to the isopropanol. Confirm that the Glycogen Solution has been added to the isopropanol of the correct tubes/samples. (Checkpoint C of DNA Extraction Worksheet).

17. Confirm the order of the samples before you transfer the supernatant from one tube to another (Checkpoint D of DNA Extraction Worksheet). Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into the corresponding 50 mL tube containing the isopropanol (or isopropanol/glycogen mixture).

18. Mix the sample by inverting gently 25 times until the white threads of DNA form a visible clump. **Note: If there is no visible pellet, the sample may be stored at room temperature in 100% isopropanol for up to 7 days. If a pellet appears, the procedure should continue as follows below (a note should be made on the worksheet and in the database regarding the extended incubation in isopropanol). If no pellet appears after 7 days, the sample will be assumed to have no measurable DNA yield and the extraction for that sample should be noted as failed.*

19. Centrifuge at 2000 x g for 3 minutes; the DNA will be visible as a white pellet.

20. Obtain a sterile 50 mL tube for each of the tubes in the extraction batch. On the tubes, write the complete family ID, 6-digit eyeGENE® ID, or simply number the tubes so that they correspond to the tubes in the batch. These tubes will be used to collect the waste from their corresponding extraction tubes in the pour-off of the isopropanol supernatant and the ethanol wash in the steps to follow. Having an individual waste tube for each tube in the extraction batch will minimize the risk of cross-contamination of samples in the event that the DNA pellet is accidentally poured off.

21. Pour off the supernatant of each sample into its corresponding waste collection tube and drain tube on clean absorbent paper. Once it has been confirmed that the DNA pellet has not been poured off into the waste tube, pour the waste from the tube into the proper chemical waste container.

22. Add 70% Ethanol (refer to the DNA sample worksheet for solution volumes as determined by starting blood volume) and invert tube several times to wash the DNA pellet.

23. Centrifuge a 2,000 x g for 1 minute (*If the pellet appears flaky, you may spin for an additional 1-5 minutes.)*.

24. Carefully pour off the ethanol into the same individually-labeled waste tubes that were previously used to collect the isopropanol supernatant. The pellet may be loose, so pour slowly and watch the pellet. Once it has been confirmed that the DNA pellet has not been poured off into the waste tube, pour the waste from the tube into the proper chemical waste container.

25. Circle the DNA pellet on the outside of the tube, and invert and drain the tube on clean absorbent paper. Allow to air dry 10-15 minutes.

DNA Hydration

26. Once dry, add DNA Hydration Solution to the tube (see Part IV, Table 1 of Extraction Worksheet for volumes).

27. Rehydrate the DNA by incubating the samples at 65°C for 1 hour, then place the samples on the shaker and incubate overnight at room temperature.

28. Confirm the order of the samples before you transfer the Hydrated DNA Solution to the 1.5mL microcentrifuge tubes (Checkpoint E of DNA Extraction Worksheet). Centrifuge the sample briefly and then transfer the predetermined amounts to their corresponding labeled 1.5 mL microcentrifuge tubes. Label the microcentrifuge tube for the negative control as “negative control” or “blank” and include the date of the extraction, the volume, and your initials.

29. Measure the DNA concentration using the Nanodrop (See Nanodrop Protocol in NEI DNA Diagnostic Laboratory SOP Manual) and enter the corresponding value on the eyeGENE® database.

    ***Note: The concentration of the negative control is acceptable if it is less than or equal to 50ng/µl. If the concentration of the negative control falls outside this range, initiate an investigation as to the cause of the abnormal concentration.*

30. Immediately place DNA at 4°C. Eventually, the samples will be frozen at -20°C. Once frozen, the tubes may be transferred to -80ºC for long-term storage. Store the negative control in the "eyeGENE® extraction negative control" box at 4ºC. All negative controls from manual extractions must be amplified by PCR to confirm that no DNA is present (Checkpoint F of DNA Extraction Worksheet). After 6 months of storage, provided that it did not amplify during PCR, the negative control will be discarded.